Antitumor Effects of a Combined 5-Aza-2'Deoxycytidine and Valproic Acid Treatment on Rhabdomyosarcoma and Medulloblastoma in Ptc1 Mutant Mice

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Abstract

Patched (Ptc1) heterozygous mice develop medulloblastoma (MB) and rhabdomyosarcoma (RMS) resembling the corresponding human tumors. We have previously shown that epigenetic silencing of the intact Ptc1 allele contributes to tumor formation in this model. Here, we investigated whether targeting of epigenetic silencing mechanisms could be useful in the treatment of Ptc1-associated cancers. A reduction of endogenous DNA methyltransferase1 (Dnmt1) activity significantly reduced tumor incidence in heterozygous Ptc1 knockout mice. A combined treatment with the Dnmt inhibitor 5-aza-2’deoxycytidine (5-aza-dC) and the histone deacetylase (HDAC) inhibitor valproic acid (VPA) efficiently prevented MB and RMS formation, whereas monotherapies with either drug were less effective. Wild-type Ptc1 expression was efficiently reactivated in tumors by 5-aza-dC/VPA combination therapy. This was associated with reduced methylation of the Ptc1 promoter and induction of histone hyperacetylation suggesting inhibition of HDACs in vivo. However, the treatment was not effective in clinically overt, advanced stage tumors. This is a first in vivo demonstration that targeting of Dnmt and HDAC activities is highly effective in preventing formation of Ptc1-associated tumors. The results suggest a novel clinical strategy for consolidation therapy of corresponding tumors in humans after completion of conventional treatment. Our data also suggest that epigenetic therapy may be less effective in treating advanced stages of tumors, at least in this tumor model. [Cancer Res 2009;69(3):887–95]

Introduction

Ptc1 is a component of a signaling pathway, which plays a major role in the control of cell differentiation and proliferation. Ptc1 is the receptor for Hedgehog (Hh) and normally inhibits its signaling partner Smoothened (Smo). Physiologic activation of the pathway is induced by Hh. Binding of Hh to Ptc1 suspends the inhibition of Smo, which leads to activation of the Gli zinc-finger transcription factors and transcriptional activation of downstream Gli target genes including Gli1 and Ptc1 itself (1). Mutational inactivation of Ptc1 results in a pathologic activation of the signaling pathway and is characterized by increased levels of Gli1 and Ptc1 mRNA (reviewed in ref. 2).

Mutations in Ptc1 have been identified in a variety of tumors including medulloblastoma (MB) and rhabdomyosarcoma (RMS; ref. 3). Although Ptc1 is assumed to act as a tumor suppressor gene, one normal allele is frequently retained in these tumors (4, 5). Using Ptc1+/-/+ mice, we recently showed that Ptc1 transcripts, which are consistently overexpressed in tumors of these mice, are derived predominantly from the mutated allele. In contrast, transcript levels of the other, mutation-free allele, seem to be unchanged or down-regulated (5, 6). Several lines of evidence suggest that the latter effect is mediated by the methylation of the wild-type Ptc1 (wtPtc1) allele: First, in breast carcinoma, Ptc1 expression is reduced due to promoter methylation (7). Second, treatment with the demethylating agent 5-azacytidine increased wtPtc1 mRNA expression in cell lines derived from MB that arise in murine Ptc1+/-/+ heterozygotes (8). Third, we have recently shown that methylation fully represses transcription from a Ptc1 promoter construct (6) and fourth, Ptc1 promoter methylation has been shown in Hh-dependent ovarian fibroma and dermoids (9), although an analysis of the same region in Hh-associated MB failed to detect any methylation changes compared with control cerebella (10).

Changes in the DNA methylation status are among the most common molecular alterations in human neoplasia (11). Best characterized are increases in DNA methylation that are associated with transcriptional silencing events particularly at promoter regions of genes that regulate important cell functions, such as tissue inhibitor of metalloproteinase 3 and MLH1 (12). The methylation of genomic DNA in malignant cells is catalyzed by DNA methyltransferases (Dnmt; ref. 12). The role of Dnmt in tumorigenesis has been impressively shown in heterozygous Dnmt1 mice, in which a 50% reduction of Dnmt1 expression was sufficient to diminish tumor numbers induced by Apc or Mlh1 mutations, or by carcinogens (13–15).

Inactivation of Dnmt leads to changes of histone modification patterns including an increase in histone acetylation (16). Therefore, a “cross talk” between DNA methylation and histone deacetylation seems to work in concert to silence gene expression. The importance of acetylation changes in Ptc1-associated tumors is unknown.
The biochemical reversibility of DNA methylating agents or histone deacetylase inhibitors has led to therapeutic application of DNA-demethylating agents and histone deacetylase (HDAC) inhibitors. One of the classic DNA-demethylating agents is 5-aza-2′-deoxycytidine (5-aza-dC), which substitutes the naturally occurring cytidine during cell division and forms a covalent bond between the DNA and the Dnmt enzyme. Both in vivo and in vitro, 5-aza-dC induces global and gene-specific DNA hypomethylation (17). 5-aza-dC is currently used in clinical studies of myelodysplastic syndrome and acute and chronic leukemia.10,11

HDAC inhibitors cause hyperacetylation of histone proteins leading to reactivation of silenced tumor suppressor genes, cell cycle arrest, apoptosis, and differentiation in a variety of cancer cells. Several HDAC inhibitors are currently being tested in clinical phase 1 and 2 studies (18, 19). One HDAC inhibitor is the short chain fatty acid valproic acid (VPA; refs. 20, 21) that has been used for many years in the treatment of epilepsy and as a mood stabilizer (22).

Due to the interplay in regulation of gene transcription, Dnmt and HDAC inhibitors exert synergistic effects in the reactivation of silent genes in tumor cells (23). It has been shown that the effects on tumor growth using a combination of the substances were superior to the treatment with either 5-aza-dC or HDAC inhibitor alone (19, 24–27).

Here, we investigated the in vivo effects of inhibition of epigenetic silencing on prevention and treatment of Ptc-h-associated tumors. For this purpose, we first assessed the effect of gene dosage of Dnmt1 on tumor development in Ptc-hneo67/+ mice. Second, we examined the possibility of pharmacologic reactivation of the wtPtc allelle by 5-aza-dC and/or VPA and its effects on tumor growth.

Materials and Methods

Compounds. 5-aza-dC and VPA were purchased from Sigma-Aldrich and diluted in Dulbecco’s PBS (PAN Biotech GmbH). For use in in vivo experiments, fresh stocks of 120 μg/ml 5-aza-dC and 320 mg/ml VPA were prepared on ice and individually diluted in PBS to the desired drug dose for each animal. Aliquots were stored for a maximum period of 2 wk at −80℃.

Experimental tumor models of Ptc-h-deficient mice. Wild-type or heterozygous Ptc-hmimRζ/+ mice were maintained on a C57BL/6 (B6) or a mixed B6×BALB/c background [hereafter B6-Ptc-hmimRζ/+ or (B6×Balb)-Ptc-hmimRζ/+ respectively] and genotyped as described (28). B6-Ptc-hmimRζ/+ mice develop MB at high incidence but are resistant to RMS formation. In contrast, a mixed B6×Balb background confers strong susceptibility to RMS but not to MB (29).

To evaluate the dependency of MB formation on DNA-methylation in a genetic experiment, B6-Ptc-hmimRζ/+ were crossed to B6;Dmnt1tm1Jae/+ mice maintained on a B6 background (hereafter Dmnt1N/−). Dmnt1N/− mice were obtained from The Jackson Laboratory and genotyped as described by Cormier and Dove (30).

All animals were treated and housed in accordance with the German animal protection laws.

Determination of the maximum tolerable dose for 5-aza-dC and VPA in B6 and B6×Balb mice. Eight-week-old B6 and B6×Balb wild-type mice (n = 10 in each cohort) were injected i.p. (150 μl per injection) with 5-aza-dC (1.6, 0.4, or 0.1 mg/kg) or VPA (640 and 400 mg/kg) daily over a period of 4 wk and followed for additional 8 wk.

The maximum tolerable dose (MTD) was defined as the dose at which all animals survived the 4-wk treatment period and the consecutive 60 d of observation, and at which overt clinical signs of toxicity were absent. Toxicity was defined as loss of body weight of >10%, general weakness, severe hair loss, or shaggy coat. In addition, blood cell counts were measured before treatment, directly after treatment, and after the observation period to detect delayed effects of the substances.

Treatment with 5-aza-dC was lethal for all mice at 1.6 mg/kg/d and for 1 mouse at 0.4 mg/kg/d, and was associated with severe reduction of leukocyte, erythrocyte, and platelet counts. Treatment with 0.1 mg/kg/d 5-aza-dC was well-tolerated and caused a moderate, reversible leukocytopenia.

Treatment with 640 mg/kg/d VPA was lethal for two mice and caused pronounced somnolence as well as leukocytopenia in all mice. In contrast, all mice survived treatment with 400 mg/kg/d VPA and leukocyte count normalized after discontinuation of therapy. Based on these data, 0.1 mg/kg/d 5-aza-dC and 400 mg/kg/d VPA were taken as the respective MTD.

For determination of the combined MTD (cMTD) and 1/2 cMTD, mice were injected daily 0.1 mg/kg/d 5-aza combined with 400 mg/kg/d VPA, or 0.05 mg/kg/d 5-aza-dC combined with 200 mg/kg/d VPA, respectively. Each drug was injected separately in a volume of 50 μl per injection to avoid premature inactivation of 5-aza-dC due to its instability in solution.

All animals survived the 4-wk application period. A reversible leukocytopenia under cMTD application was diagnosed in all animals. Treatment of the B6 strain with the cMTD resulted in pronounced somnolence, whereas the 1/2 cMTD was well-tolerated.

The cMTD (B6×Balb) and the 1/2 cMTD (B6) was also applied to 4-wk-old animals over a period of 12 wk. The reduction of blood cell count was reversible. After therapy end, the animals were observed for additional 12 wk. There were no other signs of toxicity during the treatment and the 12-wk posttreatment observation period.

Based on these observations, a cMTD of 0.1 mg/kg/d 5-aza-dC and 400 mg/kg/d VPA was applied to B6×Balb, and a 1/2 cMTD of 0.05 mg/kg/d 5-aza-dC and 200 mg/kg/d VPA was applied to B6 mice in all combination treatments.

Therapeutic treatment of tumor-bearing Ptc-hmimRζ/+ mice with 5-aza-dC/VPA. RMS-bearing (B6×Balb)-Ptc-hmimRζ/+ mice were randomized into two groups. After measurement of tumor size by volumetric computed tomography (VCT), animals were treated daily with the cMTD or PBS i.p. for 30 d. When tumors grew too large or when animals showed a poor general condition, they were euthanized and tumors were collected for molecular analysis. If possible, tumor growth of the animals was measured by VCT directly (d30) after treatment and once per month thereafter (d60, d90).

Chemopreventive treatment of Ptc-hmimRζ/+ mice with 5-aza-dC and/or VPA. Tumor-bearing (B6)-Ptc-hmimRζ/+ mice were treated for 12 consecutive weeks with either the MTD of 5-aza, the MTD of VPA, the cMTD of 5-aza-dC/VPA, or with vehicle. RMS development in (B6×Balb)-Ptc-hmimRζ/+ mice was followed by regular manual palpations of animals as most RMS develop in the muscles of the hip/thigh or intra-abdominal. Animals were either euthanized immediately after the treatment or at the age of 220 d.

Advanced stages of MB in B6-Ptc-hmimRζ/+ mice were diagnosed when the animals showed ataxia, decrease in overall activity, rough hair, weight loss, as well as signs of abnormal occipital prominence. To identify early stage tumorigenic lesions, the cerebella were removed and subjected to microscopic examination (see below).

Monitoring of RMS formation by VCT. RMS usually do not harm the animals and it is possible to monitor their growth over longer periods of time. Monitoring by noninvasive VCT using a laboratory animal fpVCT from GE Global Research was performed as described previously (31). Tumor sizes were determined according to the formula length × width × depth × 0.5 (32).

For assessment of the responsiveness of advanced stages of RMS to 5-aza-dC/VPA treatment, tumor size was measured before and directly (d30) after treatment and once a month thereafter (d60 and d90; see above).

Analysis of peripheral blood hemoglobin concentrations and leukocyte counts. Two hundred microliters of EDTA blood were collected from the retroorbital plexus before the onset of treatment, directly after the treatment period, as well as at the end of the observation period. Leukocyte,

10 http://www.fda.gov
11 http://www.supergen.com
erythrocyte, hemoglobin, and thrombocyte counts were measured with an ABX Micros 45 Hematology Analyser (ABX Diagnostics).

Histopathology. RMS and corresponding normal skeletal muscle as well as MB and cerebella were excised. Specimens used for isolation of total RNA or DNA were frozen immediately after removal. A part of each sample was embedded in paraffin for immunohistologic analysis.

For quantification of early stage MB lesions, the cerebella were subjected to serial sectioning. The identity of the tumors as RMS, MB, or as MB precursor lesions was confirmed using H&E-stained sections.

Reverse transcription and quantitative real-time PCR analysis. Total RNA was extracted from murine tissues or peripheral blood mononuclear cells (PBMC; isolated by Ficoll gradient) using TRIzol Reagent (Sigma-Aldrich). Reverse transcription was performed as previously described (6) using random hexamers and SuperScriptII Reverse Transcriptase (Invitrogen). Quantitative Real-time PCR (qRT-PCR) was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The qRT-PCR assay for Igf2 expression was the same as previously described (6). The primers and probes used for quantification of 

Table 1. Absolute numbers and percentages of symptomatic and symptom-free animals

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of mice</th>
<th>Symptomatic MB</th>
<th>Symptom-free</th>
<th>Unexplained death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnmt1+/+;Ptch&lt;sup&gt;b&lt;/sup&gt;+/+</td>
<td>27</td>
<td>17 (63%)</td>
<td>6 (22%)</td>
<td>4 (67%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dnmt1&lt;sup&gt;c&lt;/sup&gt;/+;Ptch&lt;sup&gt;b&lt;/sup&gt;+/+</td>
<td>30</td>
<td>14 (47%)</td>
<td>15 (50%)</td>
<td>6 (40%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

B. 25- to 30-d-old (B6xBalb)-Ptch<sup>b</sup>/+ mice treated with the MTD of 5-aza, the MTD of VPA or the cMTD of 5-aza-dC/VPA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Mice with palpable RMS</th>
<th>Sacrificed because of bad general condition (no RMS detected)</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>24</td>
<td>11 (46%)</td>
<td>3 (12%)</td>
<td>10 (42%)</td>
</tr>
<tr>
<td>5-aza</td>
<td>21</td>
<td>8 (38%)</td>
<td>-</td>
<td>13 (62%)</td>
</tr>
<tr>
<td>VPA</td>
<td>14</td>
<td>4 (29%)</td>
<td>-</td>
<td>10 (71%)</td>
</tr>
<tr>
<td>5-aza/VPA</td>
<td>47</td>
<td>3 (6%)</td>
<td>9 (19%)</td>
<td>35 (72%)</td>
</tr>
</tbody>
</table>

C. 25- to 30-d-old B6-Ptch<sup>b</sup>/+ mice treated with the 1/2 cMTD of 5-aza-dC/VPA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Symptomatic MB</th>
<th>Symptom-free</th>
<th>Unexplained death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>27</td>
<td>12 (44%)</td>
<td>11 (41%)</td>
<td>4 (15%)</td>
</tr>
<tr>
<td>5-aza/VPA</td>
<td>34</td>
<td>6 (17%)</td>
<td>24 (71%)</td>
<td>4 (12%)</td>
</tr>
</tbody>
</table>

NOTE: The respective Kaplan-Meier curves are shown in Figs. 1A, 2B and C.

<sup>a</sup>The percentage of mice with asymptomatic MB.
<sup>b</sup>The percentage of mice with MB precursor lesions.
<sup>c</sup>The percentage of mice with tumor-free cerebella refer to symptom-free mice.
after MeDIP, the ratios of the signals in deimmunoprecipitated DNA versus input DNA was calculated as recommended by the manufacturer.

**Statistics.** Kaplan-Meier analyses were used to assess group differences in tumor-free survival. Two-sided log-rank tests were performed. Gene expression was analyzed after a logarithmic transformation within an ANOVA setting. The required normal distribution of the residuals was verified by a Shapiro-Wilk test. P values for group effects were adjusted for multiple testing applying Dunnett's method and considering vehicle-treatment as the control group.

Changes in RMS volumes measured by VCT were analyzed after a logarithmic transformation in a linear regression model. Considering an exponential tumor growth, missing VCT data at day 30, 60, or 90 for each RMS were generated by random missing data imputation, if necessary. Statistical evaluation was performed using SAS 9.0 (SAS institute, Inc.). The level of significance was set to <0.05.

**Results**

**Reduced Dnmt1 activity delays onset of tumors in Ptch<sup>neo67/+</sup> mice.** B6-Ptch<sup>neo67/+</sup> mice were crossed with Dnmt1<sup>N</sup>/ mice, which exhibit only 50% of wild-type Dnmt1 activity (33). Because both strains were maintained on a B6 genetic background, the offspring were monitored for the formation of MB, which arise on this background (see Material and Methods section). Animals with clinical MB symptoms were sacrificed and brain tissues were subjected to histopathologic examination, as were cerebella of the symptom-free mice at the end of the observation period (250 days).

All Dnmt1<sup>+/+</sup>; Ptch<sup>1/4</sup> and Dnmt1<sup>N</sup>/; Ptch<sup>1/4</sup> mice remained healthy (data not shown). In contrast, within 250 days of life, 63% of Dnmt1<sup>1/1</sup>; Ptch<sup>neo67/+</sup> and 47% of Dnmt1<sup>N</sup>/; Ptch<sup>neo67/+</sup> mice developed symptomatic MB, which was confirmed histologically. In addition, 15% and 3% of mice with the respective genotype died for unknown reasons, whereas 22% and 50% remained healthy (numbers of animals are provided in Table 1A). The difference in MB-free survival between Dnmt1<sup>1/1</sup>; Ptch<sup>neo67/+</sup> and Dnmt1<sup>N</sup>/; Ptch<sup>neo67/+</sup> was statistically significant (P = 0.021; Fig. 1A).

Examination of cerebella of asymptomatic mice at day 250 revealed asymptomatic MB or MB precursor lesions (Fig. 1B) in 100% of mice with the Dnmt1<sup>1/1</sup>; Ptch<sup>neo67/+</sup> genotype and in 73% of Dnmt1<sup>N</sup>/; Ptch<sup>neo67/+</sup> mice (P = 0.16 by χ² test; Table 1A). Altogether, these data suggested that a 50% reduction in endogenous Dnmt1 activity delays tumor growth in heterozygous Ptch<sup>neo67/+</sup> mice.

5-aza-dC/VPA combination therapy efficiently prevents tumor formation in Ptch<sup>neo67/+</sup> mice. In addition to leukocytopenia (see Material and Methods section), the MTD of 5-aza-dC (0.1 mg/kg/d) and of VPA (400 mg/kg/d) led to a modulation of gene expression in PBMC similarly to that observed humans treated with 5-aza-dC (34) and to acetylation of H4 in the spleen, respectively (Fig. 2A). This indicates that either drug-induced changes in the expression of markers for DNA-methylation and histone acetylation.

When 25- to 30-day-old heterozygous (B6xBalb)-Ptch<sup>neo67/+</sup> mice were treated daily for 12 weeks with cMTD of 5-aza-dC/VPA or with PBS, 46% of PBS-treated, but only 6% of 5-aza-dC/VPA-treated animals developed palpable, histologically confirmed RMS (P = 0.0004; Table 1B; Fig. 2B). In contrast, monotherapy with 5-aza-dC or VPA did not result in statistically significant increase in tumor-free survival, although a positive trend was apparent (P = 0.604; Fig. 2B).

Because some animals died for unknown reasons other than RMS (see Table 1B), we reanalyzed the data by Kaplan-Meier analyses using an end point defined as absence of RMS formation or of death. Similarly to RMS-free survival, although a positive trend was apparent (P = 0.021; Fig. 1A).

To determine the effect of both drugs on MB formation, we treated B6-Ptch<sup>neo67/+</sup> mice (n = 34) with the 1/2 cMTD of 5-aza-dC/VPA. Untreated B6-Ptch<sup>neo67/+</sup> mice (n = 27) served as
controls (Table 1C). With respect to symptomatic, histologically confirmed MB, there was a significant increase in tumor-free survival in 5-aza-dC/VPA-treated animals when compared with PBS-treated controls at 115 days of life ($P = 0.017$; Fig. 2C).

**Growth of established tumors is not inhibited by 5-aza-dC/VPA.** To explore the therapeutic potential of 5-aza-dC/VPA on clinically symptomatic tumors, RMS-bearing Ptchneo67/+ mice were treated over a period of 30 days with cMTD 5-aza-dC/VPA or PBS and subjected to VCT analysis before (d0), directly after treatment (d30) and 1 and 2 months thereafter (d60, d90). Because some of the individual animals bore more than one tumor, the growth rate for each tumor was measured separately.

Throughout the experiment, neither 5-aza-dC/VPA- nor PBS-treated animals developed new tumors. Similar percentages of animals died or had to be sacrificed due to poor health in either group. As shown in Fig. 3, the difference between growth rate of the PBS- and 5-aza-dC/VPA-treated tumor was not significant at any of the time points analyzed ($P_{d30} = 0.603, P_{d60} = 0.832,$ and $P_{d90} = 0.537$) and also not for tumor growth as a function of time ($P = 0.197$).

**Histone acetylation and gene expression changes in RMS and in normal skeletal muscle in response to 5-aza-dC and VPA.** Tissues from tumor-bearing (B6xBalb)-Ptchneo67/+ mice were isolated 30 minutes to 2 hours after the last application of 5-aza-dC/VPA or PBS. In addition, samples were taken after the medication-free follow-up period of 60 days after treatment. Compared with PBS-treated RMS, acetylation of H4 was strongly induced in tissue samples from treated mice (Fig. 4A).

Up-regulation of **wtPtch** expression was significant in 5-aza-dC/VPA-treated RMS compared with PBS-treated control RMS ($P_{wtPtch} = 0.012$; Fig. 4B). This was accompanied by a moderate down-regulation of **D67Ptch** transcripts ($P_{D67Ptch} = 0.448$; Fig. 4B). The effect of 5-aza-dC/VPA treatment on **wtPtch** and **D67Ptch** expression was reversible after discontinuation of medication ($P_{wtPtch} = 0.288; P_{D67Ptch} = 0.773$). In contrast to **Ptch** transcription, **Gli1** expression was not altered in RMS. The drugs also did not influence the respective transcript levels in normal skeletal muscle (Fig. 4B).

We next evaluated the ratio of **D67Ptch** to **wtPtch** transcripts and compared them between normal skeletal muscle and RMS tissues with and without epigenetic treatment. In normal muscle, the ratio was between 0.8 and 1 in all cohorts independent of the treatment (Fig. 4C). In PBS-treated RMS, the ratio was 104 and was reduced 7-fold after treatment with 5-aza-dC/VPA ($P < 0.001$). After the treatment-free observation period the ratio increased again toward the ratio of the control group ($P = 0.366$). Altogether, these data suggest that **wtPtch** is epigenetically silenced in tumor tissue and that it is possible to effectively reactivate its expression using 5-aza-dC/VPA.

Finally, we investigated the treatment effect on the expression of **Dnmt1**, **Dnmt3a**, and **p21** mRNA. 5-aza-dC affects the

![Figure 2](https://www.aacrjournals.org/doi/fig/0008-5472.CAN-08-0946)

**Figure 2.** Tumor-free survival of **Ptch**neo67/+ mice treated with 5-aza-dC and/or VPA or PBS. Treatment of animals was started at age 4 wk. A, analysis of surrogate variables for 5-aza-dC or VPA treatment compared with PBS-treated controls. Shown is modulation of expression of selected genes in PBMC (top) or histone 4 acetylation in spleen (bottom) at therapy with the MTD of the respective drugs. Expression analysis was performed by qRT-PCR and Western blot, respectively. H2B served as a loading control for total histone content in protein extracts. B, RMS-free survival of (B6xBalb)-Ptchneo67/+ mice treated with either the MTD of 5-aza-dC, VPA, or the cMTD of 5-aza-dC/VPA or PBS. The difference between 5-aza-dC/VPA- and PBS-treated mice was significant ($P = 0.0004$). C, MB-free survival of B6-Ptch**neo67/+** mice treated with the 1/2 cMTD of 5-aza-dC/VPA or vehicle. The difference was significant compared with untreated B6-Ptch**neo67/+** mice ($P = 0.017$). B and C, shown are mice that developed clinically overt tumors within a 12-wk treatment period. The number of mice for each cohort is given in Table 1.
transcription of Dnmt1 and Dnmt3a in a cell type–depending manner (35, 36). The expression of p21 mRNA is enhanced by both 5-aza-dC and VPA (35, 37). Whereas expression analysis of Dnmt1 and Dnmt3a did not reveal significant changes in 5-aza-dC/VPA-treated RMS compared with the PBS controls (data not shown), p21 was up-regulated in RMS and skeletal muscle in 5-aza-dC/VPA-treated mice at therapy and reversed after the medication-free period. The up-regulation was significant in normal skeletal muscle \((P = 0.004, \text{Fig. 4B})\) but not in RMS \((P = 0.181)\).

**Methylation changes in 5-aza-dC–treated RMS.** The Ptch promoter contains 2 CpG islands \([\text{CpG-I1 and CpG-I2}}; \text{as defined by Takai and Jones (38)}\], CpG island are composed of >500bp with a G+C content of >65% and an observed CpG/expected CpG ratio of >0.65) and a CpG-rich region \((267 \text{bp, G+C content 55%}, \text{CpG/expected CpG ratio 0.7}; \text{Fig. 5A})\). Thus far, only CpG-I1 has been investigated for tumor-specific methylation changes, leading to conflicting results \((9, 10)\). Bisulfite sequencing of DNA derived from 5-aza-dC– or PBS-treated RMS revealed differential DNA methylation in fragment 5 in response to 5-aza-dC: Methylation was decreased in CpG 9 and 10 compared with untreated RMS (Fig. 5A). In summary, the data provide evidence that 5-aza-dC modulates the magnitude of methylation of Ptch upstream sequences.

Global DNA methylation in the same PBS– or 5-aza-dC–treated RMS was investigated by MeDIP. MeDIP revealed a >2-fold reduction in the methylation level of major satellites in RMS after treatment 5-aza-dC (Fig. 5B).

**Discussion**

This is the first in vivo study that has investigated the role of epigenetic mechanisms in the formation of Ptch-associated
tumors. It was conducted on heterozygous Ptch knockout mice, which, depending on the genetic background, spontaneously develop two important childhood tumors, MB and RMS. The results of both the genetic and the pharmacologic modulation of methylation and histone deacetylation are consistent with the involvement of these processes in the growth of tumors associated with loss of Ptch function. Importantly, we have identified one methylation-sensitive region within the murine Ptch promoter that might be responsible for the re-expression of Ptch in RMS after treatment with 5-aza-dC. As such, these results may have implications for further optimization of therapies of these tumors in children but also for other Ptch-associated cancers in adults.

A 50% reduction of endogenous Dnmt1 expression by a genetic approach resulted in a significant decrease in clinically overt MB in B6-Ptch\textsuperscript{neo67/+} mice. This was caused by a slower growth of tumors and is consistent with the well-known inhibitory effect of Ptch on cell proliferation. On the other hand, Dnmt1 deficiency had no obvious effect on the initiation of MB, as there was no statistically significant difference between Dnmt1\textsuperscript{-/-}; Ptch\textsuperscript{neo67/+} and Dnmt1\textsuperscript{-/-}; Ptch\textsuperscript{neo67/+} groups with respect to the number of animals free of early MB precursor lesions or of asymptomatic MB.

Of interest, the deficiency in Dnmt1 was not compensated in our study by any other Dnmt. Dnmt1 is considered a maintenance Dnmt, because of its high affinity for hemimethylated DNA template (39). In contrast, Dnmt3a and 3b constitute de novo methylases that affect the methylation status of normally unmethylated CpG sites (40, 41). In vitro methylation assays have shown that Dnmt3a and 3b could cooperate with Dnmt1 in the extension of methylation within the genome (42). The inability of these de novo methylases in our study to compensate for the reduced Dnmt1 activity emphasizes the attractiveness of the latter enzyme as a therapeutic target in tumor therapies.

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Methylation affects the growth of Ptch-associated tumors in concert with histone deacetylation. Although monotherapy using either 5-aza-dC or VPA was effective and diminished the number of overt tumors, a statistically significant effect was achieved only after a simultaneous inhibition of Dnmt and HDAC. This is in agreement with studies demonstrating synergistic effects of Dnmt- and HDAC inhibitors on reactivation of silent genes in tumor cells (23), on growth inhibition of leukemias (27) and Non–Hodgkin Lymphomas (19) and with our own observation that 5-aza-dC combined with VPA exert a synergistic effect on p21 expression in normal mouse muscle tissue (data not shown). The molecular mechanism of this synergy is incompletely understood but likely involves interaction of Dnmt and HDACs in mediating gene silencing (24–26).

In contrast, Dnmt- and HDAC inhibitors had no effect on the growth of advanced stages of Ptch-associated tumors. This
suggested either insufficient penetration of the drugs into the tumor or the latter’s lack of dependence on Ptch signaling. The first possibility is unlikely because wtPtch expression was efficiently reactivated in tumors by 5-aza-dC/VPA combination therapy. Incidentally, reactivation of Ptch expression and the identification of a methylation-sensitive region within the Ptch promoter provides a further confirmation of our model of tumorigenesis involving a methylation-based silencing of the wtPtch allele. The treatment was also associated with a strong induction of histone hyperacetylation, indicating inhibition of HDACs. p21 expression was basically unchanged in the tumor, perhaps due to its extremely high expression before treatment. In contrast, we observed a strong induction of p21 in the skeletal muscle, which exhibited much lower p21 basal expression.

Although wtPtch was efficiently up-regulated upon 5-aza-dC/VPA treatment, it was not sufficient to attenuate Hh signaling activity in the tumors. This was shown by unchanged Gli1 expression levels and by only moderate down-regulation of expression of the Δ67Ptch allele, the expression of which (as another downstream target of Hh signaling) should have been turned off as well. Because we observed that Δ67Ptch inhibits wtPtch function in a dominant-negative manner,12 lack of pathway inhibition might be mediated by the ratio of Δ67Ptch/wtPtch transcript levels, which never reached the levels in normal tissue.

Alternatively, advanced stages of tumors may have become independent on Ptch signaling. This result would be in agreement with our recent data with cyclopamine, which binds and inhibits Smo and subsequently blocks Hh pathway activity. Cyclopamine works in the prevention and treatment of early tumor stages (43) but fails to inhibit growth of advanced stages tumors in Ptcgh−/− mice (44).

Although the mechanism underlying the success of epigenetic therapy in the prevention of RMS and MB in Ptcgh−/− mice may be in part independent from Hh/Ptch signaling and requires further investigations, the study provides important results with respect to novel treatment options of PTCH-associated tumors in humans. Specifically, epigenetic therapy could be effectively used for prevention of tumor formation or for controlling minimal residual disease after conventional therapy, to reduce relapse rates and treatment failure. For example, in childhood MB, epigenetic therapy could play a role as consolidation therapy within a multimodal treatment concept after the bulk tumor has been removed by surgery and radiation. Because sporadic MB as well as BCC frequently show mutations in, or loss of one PTCH allele, whereas the other PTCH allele seems to be normal (45–48), it is possible that silencing of the normal PTCH allele is also involved in the process of tumor formation in humans. Thus, our studies might provide a new avenue for therapies of tumor entities showing an epigenetic inactivation of the tumor suppressor gene PTCH.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Antitumor Effects of a Combined 5-Aza-2′Deoxycytidine and Valproic Acid Treatment on Rhabdomyosarcoma and Medulloblastoma in Ptch Mutant Mice

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